Novel Mammalian Group XII Secreted Phospholipase A_2 Lacking Enzymatic Activity[†,‡]

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ABSTRACT: An increasing number of mammalian secreted phospholipases A_2 (sPLA$_2$s) has been identified over the past few years. Here, we report the identification and recombinant expression of a novel sPLA$_2$-like protein in mouse and human species that has been called group XIIB (GXIIB). The mature protein has a molecular mass of 19.7 kDa and structural features similar to those of the previously identified GXII sPLA$_2$, now called GXIIA. Strikingly, the GXIIB sPLA$_2$ has a mutation in the active site, replacing the canonical histidine by a leucine, suggesting that this sPLA$_2$ is catalytically inactive. Recombinant expression of human (hGXIIB) and mouse (mGXIIB) sPLA$_2$s in *Escherichia coli* indicates that GXIIB sPLA$_2$s display no measurable lipolytic activity on various types of phospholipid substrates. Furthermore, these sPLA$_2$-like proteins display relatively weak affinity to phospholipid vesicles. Binding experiments indicate that these proteins are also unable to bind to the well-known M-type sPLA$_2$ receptor. The RNA tissue distribution of GXIIB sPLA$_2$s is distinct from that of other sPLA$_2$s including the homologous GXIIA. Strong expression was observed in liver, small intestine, and kidney in both human and mouse species. Interestingly, the expression of the novel sPLA$_2$ is dramatically decreased in human tumors from the same tissues. The absence of enzymatic activity suggests that the GXIIB sPLA$_2$-like proteins probably exert their biological roles by acting as ligands for as yet unidentified receptors.

Phospholipases A_2 (PLA$_2$s) are enzymes that catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position to release free fatty acids and lysophospholipids (*1*−*4*). Over the past few years, it has been realized that PLA$_2$s constitute in humans a superfamily of enzymes comprising at least a set of 10 distinct intracellular enzymes and another set of 10 different extracellular, i.e., secreted PLA$_2$s (sPLA$_2$s). This is in marked contrast with the phospholipase C and D families that only comprise intracellular forms (*5*−*8*). The sPLA$_2$ family can be further subdivided into three main structural collections (*1*, *4*, *9*, *10*). sPLA$_2$s IB, IIA, IIC, IID, IIE, IIF, V, X, and otoconin-95 belong to the I/II/V/X sPLA$_2$ collection, while sPLA$_2$s III and XII are unique members of the group III and XII sPLA$_2$ collections. All of the sPLA$_2$s have no sequence homology with the intracellular PLA$_2$s but share among each other a number of common properties including a relatively low molecular mass of 14−19 kDa (except for GIII sPLA$_2$), a large number of disulfides, and a similar Ca^{2+}-dependent catalytic mechanism (*3*).

Collectively, mammalian PLA$_2$s are proposed to play a key role in various biological processes, including homeostasis of cellular membranes, lipid digestion, host defense, and production of potent lipid mediators such as eicosanoids and lysophospholipids, which in turn exert a wide variety of functions under normal and pathological conditions (*1*, *2*, *4*, *11*, *12*). Studies with knock-out mice and specific inhibitors have clearly demonstrated that the cytosolic PLA$_2$ IVA plays a major role in the production of lipid mediators under both normal and disease conditions (*12*−*21*). On the other hand, the specific biological functions of the different sPLA$_2$s are still unclear, despite a number of studies suggesting that sPLA$_2$s contribute to host defense and lipid mediator release, especially in inflammation and its associated diseases and in various cancers (*1*, *4*, *11*, *22*−*28*). Clearly, the different mammalian sPLA$_2$s are not isoforms because they all share a low level of sequence identity (as low as 15%) and have specific tissue distribution patterns, suggesting distinct biological roles for each of them (*29*−*31*). Furthermore, the recent analysis of their enzymatic properties has revealed dramatic differences in terms of their specific activities and substrate selectivity (*32*), again arguing for distinct functions. Finally, the identification of specific sPLA$_2$ receptors in mammals has opened the possibility that sPLA$_2$s function not only as enzymes but also independently of their catalytic activity by acting as ligands, as is the case for hormones and cytokines (*23*, *33*).

Besides mammals, sPLA$_2$s are also present in many other genomes, indicating that some of the sPLA$_2$ functions may have been conserved throughout evolution. Indeed, several

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[‡] The nucleotide sequences reported in this paper have been deposited in the GenBank/EBI Data Bank with accession numbers AY358032 (hGXIIB) and AY358033 (mGXIIB).

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$sPLA_2$s have been identified in other vertebrates including reptiles and fish, in invertebrates including *Drosophila* and worms, as well as in plants, viruses, and procaryotes (*1*, *2*, *34*, *35*). $sPLA_2$s are also particularly abundant in snake venoms, and up to 15 distinct $sPLA_2$s can be found in a single snake venom (*36*−*39*). More than 400 $sPLA_2$ sequences have now been reported from snake, insect, and molluscan venoms. Besides digestion, these $sPLA_2$s serve toxic functions that are not directly correlated with their catalytic activity. Interestingly, several natural $sPLA_2$ variants with deletions or point mutations in the active site have been described in snake venoms (*37*, *39*). Remarkably, such $sPLA_2$s are still toxic, though they are devoid of $sPLA_2$ activity.

In the past few years, a systematic screening of the nucleic acid databases generated by the genome projects using all known $sPLA_2$ structures has allowed us to identify and clone several novel mammalian $sPLA_2$s (*9*, *29*, *30*, *40*−*42*). Within our continuous search for novel mammalian $sPLA_2$s, we now report the molecular characterization of the mammalian group XIIB $sPLA_2$. Strikingly, this enzyme represents the first typical mammalian $sPLA_2$ that is expressed in many tissues under normal and pathological conditions and that is clearly catalytically inactive because of a single point mutation in the active site and altered phospholipid-binding properties.

EXPERIMENTAL PROCEDURES

Molecular Cloning of hGXIIB and mGXIIB $sPLA_2$s. Searching for $sPLA_2$ homologues in genomic databases using the tBLASTn program and known venom and mammalian $sPLA_2$ sequences as queries resulted in the identification of novel GenBank entries from different species (for example, AI657758, AI958157, AI530426, BF196453, and BY708023). Among these entries, three human and mouse entries (AF349540, AF339053, and AF339738) were reported to code for a putative novel $sPLA_2$ that was tentatively submitted as group XIII $sPLA_2$. However, because of its typical group XII structural properties, this $sPLA_2$ will be referred hereafter as GXIIB $sPLA_2$. To isolate by RT-PCR[1] the full-length open reading frame coding for this novel $sPLA_2$ in mouse and humans, we first designed two sets of forward and reverse primers flanking the coding sequences. These primers contained appropriate restriction sites for the subsequent cloning into the mammalian expression vector pRc/CMVneo (Invitrogen). Forward and reverse mouse primers were 5′-TTT GCG GCC GCC ATG AAG CTG CTC TGC GGC TTC T-3′ and 5′-AAA TCT AGA ATT CAT AGC TCT TCT TTC TCC TCC T-3′. Forward and reverse human primers were 5′-TTT GCG GCC GCC ATG AAG CTG GCC AGT GGC AAC T-3′ and 5′-AAA TCT AGA ATT CAT AAC TCT TCC TTC TCC TCC T-3′). The expected PCR fragment coding for mGXIIB was amplified from pooled cDNA from mouse embryos, small intestine, liver, colon, and lymph node tissues (BD Clontech), while that coding for hGXIIB was amplified from human liver cDNA (BD Clontech). PCR was performed for 35 cycles of amplification (94 °C/30 s, 60 °C/30 s, 72 °C/60 s) using a proofreading Taq/Pwo DNA polymerase mixture (Hybaid). The PCR fragments were digested with *Not*I and *Xba*I and ligated into the mammalian expression vector pRc/CMVneo, and several clones were entirely sequenced. All of the mouse clones were found to be identical to the open reading frame of the submitted mouse sequence GenBank AF339738. On the other hand, two sets of human clones differing at nucleotide position 464 (relative to the open reading frame) were found. The first set contains an open reading frame of 585 nucleotides that is identical to the submitted human sequence GenBank AF349540. The second set contains an open reading frame of 588 nucleotides identical to the first set, but with a three nucleotide insertion coding for an extra alanine residue, that matches the second submitted human sequence GenBank AF339053 (see also Figure 1). We also designed two other sets of mouse and human primers flanking the longest consensus nucleotide sequences obtained by alignment of the various ESTs and other sequences found in the nucleotide databases. These primers were used for PCR experiments as above to isolate these longer sequences. The DNA fragments corresponding to these consensus nucleotide sequences could be amplified from human kidney and mouse embryo cDNA and were entirely sequenced on both strands. These sequences have been deposited in GenBank with the accession numbers AY358032 (hGXIIB) and AY358033 (mGXIIB).

Molecular Cloning of mGXIIA. A forward primer (5′-TTT GCG GCC GCC ATG GTG ACT CCG CGG CGG CCC GCG CCC-3′) and a reverse primer (5′-AAA TCT AGA GAA TTC ATA GAT CTG TTT TTT CTT CAT AAC-3′) flanking the open reading frame of mGXIIA (*10*) and containing appropriate restriction sites were used to amplify by RT-PCR the sequence coding for mGXIIA. The expected fragment of 579 nucleotides was amplified from mouse liver cDNA (BD Clontech) and subcloned into the pRc/CMVneo vector as described above. The sequence was found to be identical to that published by Ho et al. (*10*).

Recombinant Expression of mGXIIA, mGXIIB, and hGXIIB $sPLA_2$s in Escherichia coli. Mature GXIIA and GXIIB $sPLA_2$s (i.e., without their signal peptide) were produced in *E. coli* as C-terminal fusion proteins with a truncated glutathione *S*-transferase (8.7 kDa) encoded by the pAB3 vector (*41*). This vector has previously been used in our laboratories to produce various $sPLA_2$s in *E. coli* (*32*) and was originally derived from the pGEX-2T vector (*43*). To subclone the $sPLA_2$s into the pAB3 vector, PCR reactions were performed as above using the pRc/CMVneo constructs as templates, the above reverse primers and forward primers containing a *Bam*HI site, and a factor Xa cleavage site (Ile-Glu-Gly-Arg) followed by the predicted N-terminal mature sequences of mGXIIA, mGXIIB, and hGXIIB (mGXIIA, 5′-TTT GGA TCC ATC GAA GGT CGT CAG GAA CAG GAC CAG ACC ACC GAC-3′; mGXIIB, 5′-TTT GGA TCC ATC GAA GGT CGT GAC CCC AGC CCC AAG GAA GAG-3′; hGXIIB, 5′-TTT GGA TCC ATC GAA GGT CGT CAG AGC GAC ACG AGC CCT GAC A-3′). The PCR

[1] Abbreviations: EST, expressed sequence tag; RT-PCR, reverse transcription−polymerase chain reaction; MALDI-TOF, matrix-assisted laser desorption ionization−time of flight; DoetPC and DoetPS, 1,2-dioleyl-*sn*-glycero-3-phosphocholine and -phosphoserine, respectively. A comprehensive abbreviation system for the various secreted phospholipases A_2 ($sPLA_2$s) is used: each $sPLA_2$ is abbreviated with a lowercase letter indicating the $sPLA_2$ species (h and m for human and mouse) that is followed by capital characters identifying the $sPLA_2$ group (GIB, GIIA, GIIC, GIID, GIIE, GIIF, GIII, GV, GX, GXIIA, and GXIIB for group IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA, and XIIB, respectively).

products were digested with *Bam*HI and *Hin*dIII and subcloned in-frame with the truncated GST protein in the pAB3 vector. All the constructs were entirely sequenced.

Protein expression was performed in *E. coli* BL21 host cells grown in Terrific broth containing ampicillin (100 μg/mL). Cells were grown in 2 L flasks to OD_{600} ~0.8 and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside overnight at 37 °C. Cells from a 600 mL culture were pelleted, solubilized in 40 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 1% Triton X-100, and 1% sodium deoxycholate) for 1 h at 4 °C, and homogenized by two passages in a French press (SLM Aminco, maximum pressure 1500 psi). Inclusion bodies were collected by centrifugation at 10000*g* for 20 min and washed twice with lysis buffer without detergent. The resulting pellet was solubilized in 40 mL of 50 mM Tris-HCl, pH 8.0, 8 M urea, 0.1 M NH_4Cl, and 0.3 M Na_2SO_3. The solubilized protein was sulfonated by addition of 0.2 volume of Thannhauser's reagent (*44*) for 1 h at room temperature. After overnight dialysis at 4 °C against 8 L of 1% acetic acid with two buffer changes, the precipitated proteins were pelleted and resuspended at 0.2 mg/mL in 500 mL of 50 mM Tris-HCl, pH 8.0, 8 M urea, and 0.1 M NH_4Cl. The denatured $sPLA_2$ was refolded by dialysis against 8 L of 50 mM Tris-HCl, pH 8.0, 2 M urea, 0.1 M NH_4Cl, 5 mM $CaCl_2$, 5 mM L-cysteine, and 5 mM L-methionine for 2−4 days at 4 °C. The refolded mixture was filtered through a layer of Sephadex G-50 to remove large aggregates and subjected to extensive digestion at room temperature with 400 units of factor Xa (Amersham Biosciences). The time course of cleavage (up to 3 days) was followed by $sPLA_2$ activity (mGXIIA) or MALDI-TOF mass spectrometry (hGXIIB and mGXIIB). The protein solution was concentrated to 20 mL by ultrafiltration using an Amicon stirred cell concentrator with a YM-10 membrane, and the buffer was then exchanged to 1% acetic acid (10% acetonitrile was added for GXIIB $sPLA_2$s). The solution was filtered and loaded onto a Spherogel TSK SP-5PW column (10 μm, 7.5 × 75 mm, Tosoh Biosep) equilibrated in 1% acetic acid (and 10% acetonitrile for GXIIB $sPLA_2$s). The column was eluted at 1 mL/min using a linear gradient of ammonium acetate (0−2 M, pH 6.8, over 100 min) in 10% acetonitrile. The fractions containing cleaved $sPLA_2$ were pooled, lyophilized, resuspended in 10% acetonitrile and 0.1% TFA, and applied to a reverse-phase HPLC column (C18 Nucleosil 4.6 × 250 mm, 300 Å, 5 μm, Macherey-Nagel). Elution was performed at 1 mL/min using a gradient of acetonitrile in 0.1% TFA (10−30% acetonitrile over 20 min, then 30−35% acetonitrile over 25 min, and 35−60% acetonitrile over 30 min). mGXIIA, mGXIIB, and hGXIIB proteins appear more than 99% pure when analyzed by SDS−polyacrylamide gel electrophoresis and MALDI-TOF mass spectrometry. MALDI-TOF analysis of $sPLA_2$s was carried out on an Applied Biosystems voyager DE-Pro spectrometer under linear mode and using sinapinic acid as a matrix. Analysis of pure proteins was performed with internal calibration using appropriate protein standards (see Figure 3).

Enzymatic Activity of Group XII sPLA$_2$s and Interfacial Binding to Vesicles. Enzymatic activity of GXII $sPLA_2$s was analyzed using [^{3}H]oleate-radiolabeled *E. coli* membranes (*40*), pyrene−phosphatidylglycerol assays (*32*), and the fatty acid binding protein assay using dioleoylphosphatidylcholine vesicles as substrate (*45*). *E. coli* enzymatic activity assays were performed at room temperature for 1 h in 100 μL of $sPLA_2$ activity buffer (0.1 M Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 0.1% bovine serum albumin) containing 100000 dpm of [^{3}H]oleate-radiolabeled *E. coli* membranes. Reactions were stopped by addition of 300 μL of stop buffer (0.1 M EDTA, pH 8.0, 0.1% fatty acid-free bovine serum albumin) and centrifuged at 10000*g* for 5 min, and the supernatants containing released [^{3}H]oleate were counted.

Binding studies with GXII $sPLA_2$s on sucrose-loaded unilamellar vesicles of diether phospholipids were performed as described (*45*). Binding assays were carried out in 5 mM MOPS, pH 7.4, 0.1 M KCl, and 2 mM $CaCl_2$ at room temperature using the centrifugation method in which the amount of $sPLA_2$ remaining in the supernatant above pelleted vesicles is quantified by either enzymatic assay (GXIIA) or protein quantification by Bradford assay (GXIIB). Binding experiments with GXIIA $sPLA_2$s were carried out with 0.5 μg of pure enzyme in 100 μL of binding buffer, whereas those with GXIIB $sPLA_2$s were carried out with 400 μL of binding buffer and 2 μg of pure proteins.

M-Type Receptor Binding Studies. Competition binding assays between unlabeled GXIIB $sPLA_2$s and iodinated OS_1 to recombinant mouse M-type receptor expressed in COS cells were performed as described (*46*). For direct binding experiments with iodinated mGXIIB, mGXIIB was iodinated with lactoperoxidase (*47*) and purified by reverse-phase HPLC as above, and the labeled $sPLA_2$ was analyzed for its binding properties to recombinant mouse M-type receptor. Briefly, membranes from COS cells containing mouse M-type receptor, labeled $sPLA_2$ ligand (OS_1 or mGXIIB), and unlabeled $sPLA_2$s were incubated at 20 °C in 1 mL of binding buffer (140 mM NaCl, 0.1 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin). Incubations were initiated by addition of membranes and filtered after 60 min through GF/C glass fiber filters presoaked in 0.5% poly(ethylenimine).

Analysis of the Tissue Distribution of Mouse Group XII sPLA$_2$s. A mouse northern blot (BD Clontech, catalog no. 7762-1) and a mouse RNA master blot (BD Clontech, catalog no. 7771-1) were successively hybridized with ^{32}P-labeled mGXIIB and mGXIIA antisense riboprobes in ULTRAHyb hybridization buffer (Ambion, catalog no. 8670) for 18 h at 70 °C. High-sensitivity strippable riboprobes corresponding to the sequence of mature $sPLA_2$s were synthesized using the Strip-EZ RNA Ambion kit (catalog no. 1360) and purified using quick spin columns for radiolabeled RNA purification (Roche Applied Sciences). Blots were washed to a final stringency of 0.1× SSC (30 mM NaCl, 3 mM trisodium citrate, pH 7.0) in 0.1% SDS at 70 °C and exposed to Kodak Biomax MS films with a Transcreen-HE intensifying screen. After hybridization, blots were stripped as specified in the Strip-EZ RNA Ambion kit, checked for efficient destripping, and hybridized with the next $sPLA_2$ riboprobe. The absence of cross-hybridization with other mouse $sPLA_2$s was confirmed by parallel hybridization of Southern blots containing the 11 full-length mouse $sPLA_2$ cDNAs under identical conditions (not shown).

Analysis of the Tissue Distribution of Human Group XII sPLA$_2$s and Cancer Profiling Array. A human northern blot (BD Clontech, catalog no. 7780-1) and a Cancer Profiling

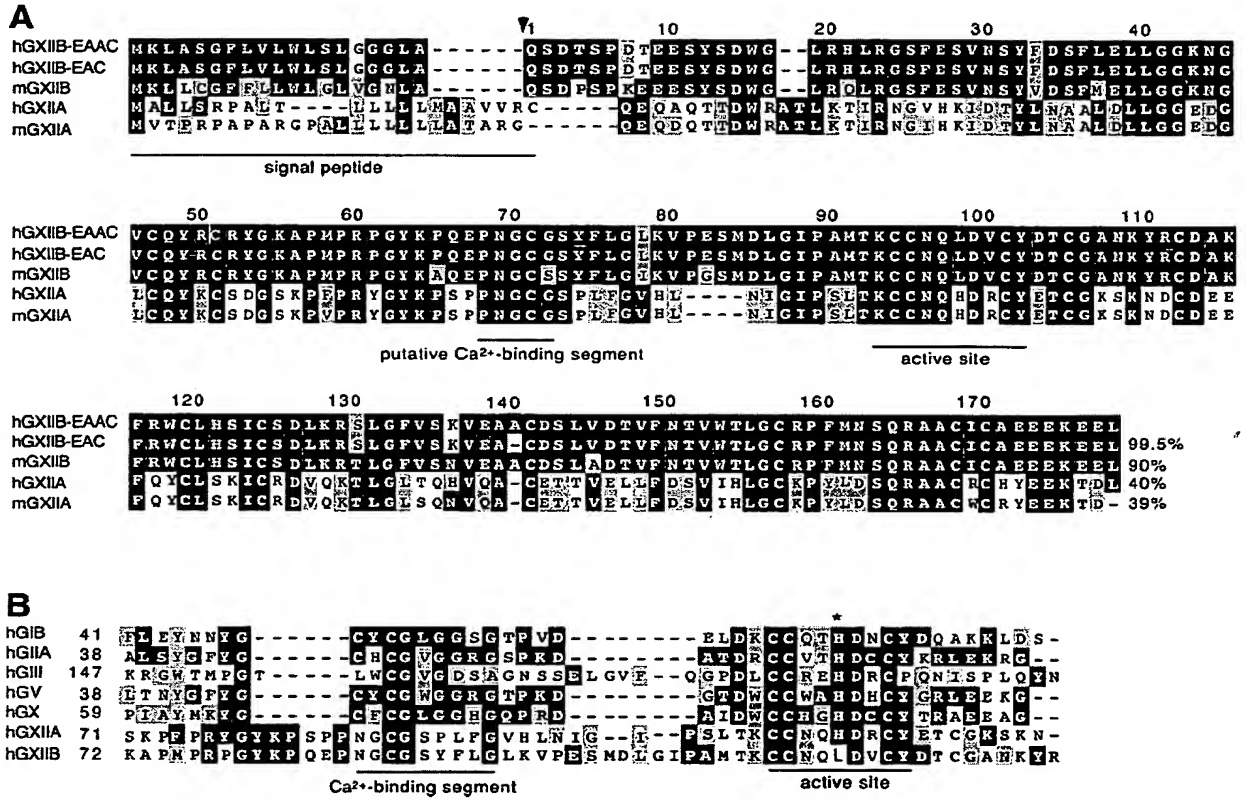

FIGURE 1: Alignment of the amino acid sequences of group XII sPLA$_2$s and other sPLA$_2$s. (A) Alignment of group XII sPLA$_2$s. hGXIIB EAAC differs from hGXIIB EAC at position 140. The mGXIIA sequence shown corresponds to the splice variant 1 from ref *10*. The arrowhead indicates the predicted signal peptide cleavage site (*49*). The active site region containing catalytic site residues and the putative Ca^{2+}-binding segment are indicated. The level of identity of each sPLA$_2$ to hGXIIB EAAC is indicated. (B) Alignment of the Ca^{2+}-binding and active site regions of hGXIIB with a representative member of the different structural groups of mammalian sPLA$_2$s.

array II (BD Clontech, catalog no. 7847-1) were successively probed with hGXIIB and hGXIIA riboprobes as described above, except that the cancer profiling array containing spotted cDNA was hybridized at 42 °C and washed to a final stringency of 0.1× SSC in 0.1% SDS at 42 °C. The absence of cross-hybridization of the sPLA$_2$ riboprobes with the other human sPLA$_2$s was checked by performing parallel hybridization of Southern blots containing the 10 full-length human sPLA$_2$ cDNAs under identical conditions (not shown).

RESULTS AND DISCUSSION

Molecular Cloning of a New Member of Group XII sPLA$_2$. During the past few years, the search for protein homologues in genomic databases fueled by the genome sequencing projects by using the tBLASTn sequence alignment program (in silico cloning) has been a very useful approach to identify novel sPLA$_2$s. This strategy has led to the cloning in our laboratory of hGX and mGX sPLA$_2$s (*29*, *40*), mGIID, mGIIE, mGIIF, and hGIIF sPLA$_2$s (*29*, *30*, *41*), hGIII (*42*) and mGIII sPLA$_2$s (S. Bezzine and G. Lambeau, unpublished data), and hGXII (hereafter called hGXIIA) sPLA$_2$ (*9*). hGXIIA and mGXIIA were independently cloned by Ho and colleagues (*10*). Using the same in silico cloning strategy, hGIID and hGIIE sPLA$_2$s were cloned by Hanasaki and colleagues (*31*, *48*).

During the identification of hGXIIA, we noticed that genomic databases contain several ESTs coding for a zebrafish group XII sPLA$_2$ (GenBank AI657758, AI397414, AI878221, among others) with a leucine in place of histidine in the active site (*9*). This sPLA$_2$ had a low sequence identity with hGXIIA, much lower than that observed between hGXIIA and mGXIIA or even with GXIIA from *Xenopus laevis* (*9*), suggesting the existence of a possible second group XII sPLA$_2$ member (hereafter called GXIIB sPLA$_2$). Within our continuous searching in databases, we then found several GenBank releases for ESTs coding for partial sequences of human and mouse forms of this sPLA2 (GenBank BF196453 and AI530426, for example). More recently, we found the releases for a putative human and mouse sPLA$_2$ that was tentatively called group XIII (GenBank AF349540, AF339053, and AF339738) and that corresponds in fact to the above group XIIB sPLA$_2$. The human and mouse protein sequences deduced from all of the nucleic sequences were found to be highly homologous to the previously identified protein sequence obtained from the alignment of the zebrafish ESTs, but they were much less homologous to mouse and human group XIIA sPLA$_2$s (*9*, *10*). Because these new protein sequences had obvious structural features of group XIIA sPLA$_2$s, we called them group XIIB sPLA$_2$s (Figure 1). Using all of the above sequence information available, a consensus nucleic acid sequence of 1069 nucleotides containing 67 nucleotides of the 5′ noncoding sequence, an open reading frame of 588 nucleotides, and 414 nucleotides of the 3′ noncoding

sequence was compiled for hGXIIB $sPLA_2$. Similarly, a nucleic acid sequence of 1078 nucleotides with 5′ and 3′ noncoding sequences of 115 and 375 nucleotides and an open reading frame of 588 nucleotides was obtained for mGXIIB.

To experimentally verify the identity of these sequences and isolate the cDNAs coding for hGXIIB and mGXIIB, we first designed specific primers flanking the complete open reading frames and used these primers in RT-PCR experiments on various human and mouse cDNAs. The expected cDNAs were amplified from human liver cDNA and pooled cDNAs from various mouse tissues (see Experimental Procedures). Sequencing of the PCR fragments revealed the correct sequences for both hGXIIB and mGXIIB. However, a number of hGXIIB clones were found to contain a deletion of three nucleotides coding for an alanine at nucleotide position 464 (amino acid position 140 in Figure 1). Further analysis of the databases revealed that the genes for hGXIIB and mGXIIB $sPLA_2$s consist of at least 4 exons and 5 introns spanning about 20 kilobase pairs. The alanine deletion above was found to occur at an intron−exon junction, suggesting that this deletion results from uncorrect splicing of the primary RNA transcript. We also designed primers flanking the longest consensus sequences obtained above for hGXIIB and mGXIIB. The corresponding DNA fragments could be amplified from human kidney and mouse embryo and were entirely sequenced on both strands. These sequences have been deposited in GenBank with the accession numbers AY358032 (hGXIIB) and AY358033 (mGXIIB).

The genes for hGXIIB and mGXIIB were found to be located on human and mouse chromosome 10, i.e., on a chromosome distinct from those where hGXIIA (chromosome 4) and mGXIIA (chromosome 3) have been identified (*9*). Finally, searching the nucleic acid databases for orthologues of GXIIB $sPLA_2$ in different species revealed that this $sPLA_2$ is present in various mammals (rat, ox, hog), birds (chicken), fishes (zebrafish, salmon), and amphibians (frog). No evidence for an orthologue of GXIIB was found in invertebrates including *Drosophila melanogaster* and *Caenorhabditis elegans* and in plants.

Structural Features of Group XIIB $sPLA_2$s. Similar to other $sPLA_2$s, the open reading frame of GXIIB $sPLA_2$s begins with a canonical signal peptide of 19 residues (Figure 1), as predicted from Nielsen et al. (*49*). This signal peptide appears to be shorter than those of GXIIA $sPLA_2$s (Figure 1). The mature hGXIIB (with the EAAC sequence) and mGXIIB proteins consist of 176 residues with calculated molecular masses of 19727.36 and 19402.04 Da. An acidic p*I* value of 5.16 was calculated for both proteins. No putative sites of N-glycosylation were found in the sequences.

Sequence comparison revealed that the level of identity between hGXIIB and mGXIIB is very high (90%), as previously observed between hGXIIA and mGXIIA [94% (*9*)]. Interestingly, this level of interspecies identity is much higher than that found for the different $sPLA_2$s from the I/II/V/X collection (*29*, *30*). On the other hand, only about 40% identity is found between GXIIB and GXIIA for both mouse and human $sPLA_2$s, clearly indicating that these $sPLA_2$s are distinct. Fifty-seven amino acids are identical between the two proteins. Of these residues, 14 are cysteines and several are found within the Ca^{2+}-binding segment and the active site. Most importantly, the two proteins differ in the active site, with the presence of a leucine in GXIIB instead of the

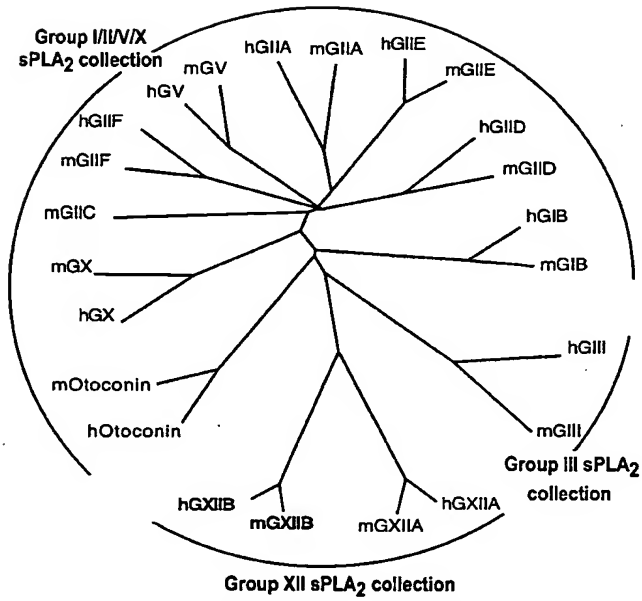

FIGURE 2: Sequence identity dendrogram of mammalian $sPLA_2$s. Sequences of mature $sPLA_2$ proteins or $sPLA_2$ domains of otoconin-95 and group III $sPLA_2$s were aligned using Clustal W, and the dendrogram was generated with Treeview. The $sPLA_2$ sequences have been retrieved from GenBank accession numbers.

canonical histidine found in GXIIA and all other $sPLA_2$s (*1*, *2*), suggesting that GXIIB $sPLA_2$s might be catalytically inactive enzymes (see below). As shown in Figure 1B, human GXIIB $sPLA_2$ has a low sequence homology with the other groups of human $sPLA_2$s, and this homology is limited to the Ca^{2+}-binding segment and the active site. Finally, on the basis of the dendrogram shown in Figure 2, it is clear that GXIIB $sPLA_2$s belong to the group XII $sPLA_2$ collection (*1*, *9*).

Recombinant Expression of Group XIIB $sPLA_2$s. To determine whether the novel mGXIIB and hGXIIB (EAC and EAAC) $sPLA_2$s are catalytically active, we first transiently transfected HEK293 cells (*41*) with the mammalian expression vector pRc/CMVneo constructs containing the full-length human and mouse GXIIB cDNAs (see Experimental Procedures). No $sPLA_2$ activity could be detected with the sensitive $sPLA_2$ assay using radiolabeled *E. coli* membranes in cell supernatant or lyzed cells between 1 and 5 days after transfection (not shown). In comparison, weak, but significant $sPLA_2$ activity could be measured when cells were transfected with the pRc/CMVneo constructs for hGXIIA and mGXIIA (not shown). We therefore expressed the GXIIB proteins in *E. coli* using the pAB3 expression system that has been previously used in our laboratory to produce several mammalian $sPLA_2$s including hGXIIA (*9*, *32*, *41*). Human and mouse group XIIA $sPLA_2$s were also produced in parallel. With this expression system, GXII $sPLA_2$s are produced as insoluble fusion proteins with the ~8 kDa N-terminal fragment of glutathione *S*-transferase, and a factor Xa protease site is located before the N-terminal residue of mature $sPLA_2$s. After in vitro refolding of inclusion bodies, the fusion proteins are cleaved with factor Xa and purified to homogeneity by cation-exchange and reverse-phase HPLC. The purified $sPLA_2$s migrate as pure proteins of the expected size on a Laemmli SDS gel (Figure

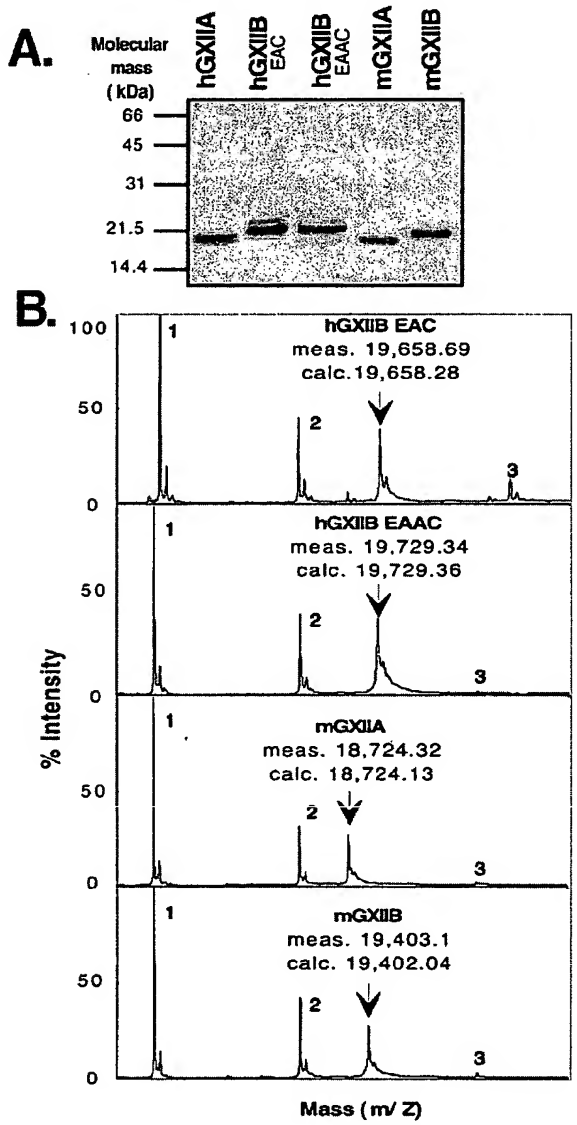

FIGURE 3: Analysis of recombinant group XII $sPLA_2$s by SDS–PAGE and MALDI-TOF mass spectrometry. (A) One microgram of purified recombinant GXII $sPLA_2$s was loaded on a 14% SDS–polyacrylamide gel under reducing conditions and stained with Coomassie brilliant blue. (B) Three picomoles of purified recombinant proteins was analyzed by MALDI-TOF mass spectrometry using internal calibration. Horse heart cytochrome *c* (MM = 12361.12 Da, peak 1), horse heart apomyoglobin (MM = 16952.5 Da, peak 2), and bovine trypsinogen (MM = 23981.98 Da, peak 3) were used as internal standards. Measured and calculated molecular masses of each $sPLA_2$ are indicated.

3A). Indeed, hGXIIA and mGXIIA migrate slightly faster than hGXIIB and mGXIIB $sPLA_2$s, in agreement with their calculated molecular masses (Figure 3B). To establish that all of the disulfide bonds are formed in the refolded $sPLA_2$s, we determined the molecular masses of the purified $sPLA_2$s by MALDI-TOF mass spectrometry with internal calibration (Figure 3B). For all $sPLA_2$s, we measured molecular masses which are different from the calculated molecular masses by less than 1 amu. These data established that the disulfide bonds are formed in the refolded $sPLA_2$s and that the proteins have not been modified during their production.

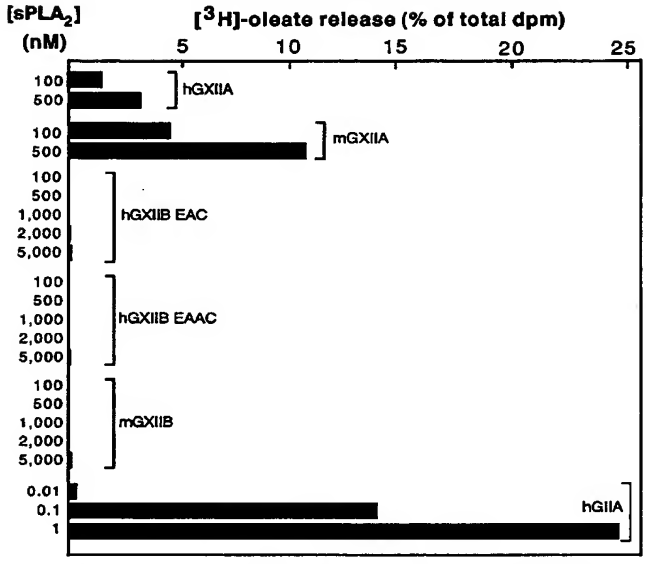

FIGURE 4: Comparison of the catalytic activity of group XIIA and XIIB $sPLA_2$s. The enzymatic activity of recombinant $sPLA_2$s was measured using labeled *E. coli* membranes as described in Experimental Procedures. $sPLA_2$s were incubated at the indicated concentrations for 1 h at room temperature with 100000 dpm of radiolabeled membranes. Values are expressed as the percentage of [^{3}H]oleic acid specifically released by $sPLA_2$s. The average amount of [^{3}H]oleic acid released in the absence of $sPLA_2$ was 6400 dpm. No significant $sPLA_2$ catalytic activity was measured for GXIIB $sPLA_2$s. Recombinant hGIIA and hGXIIA were prepared as described (*32*). Note that hGIIA is about 1000-fold more active than GXIIA $sPLA_2$s.

Enzymatic Properties of Recombinant Group XIIB $sPLA_2$s. We first analyzed the enzymatic activity of the refolded GXIIB $sPLA_2$s with the radiolabeled *E. coli* assay (*40*). At concentrations as high as 5 μM, no significant $sPLA_2$ activity could be observed for mGXIIB and the two forms of hGXIIB proteins, indicating that GXIIB $sPLA_2$s are indeed catalytically inactive (Figure 4). Using the same assay conditions, hGXIIA and mGXIIA were able to release a significant amount of radiolabeled [^{3}H]oleic acid at lower concentrations of 100 and 500 nM (Figure 4), despite the fact that these enzymes have been reported to already have very low specific activities (*9*, *32*). For comparison, we found that, in the same assay conditions, 1 nM human group IIA $sPLA_2$ (hGIIA) could hydrolyze up to 25% of the substrate. The three GXIIB proteins were also found to be inactive when anionic vesicles of pyrene–phosphatidylglycerol (*32*) or dioleoylphosphatidylcholine vesicles (*45*) were used (not shown).

We also analyzed the binding properties of GXIIB $sPLA_2$s to phospholipid vesicles composed of 70% DoetPC and 30% DoetPS (diether, nonhydrolyzable phospholipids). These types of vesicles are known to tightly bind various $sPLA_2$s (*32*). With or without added Ca^{2+} in the binding assays, very low dissociation constants (K_d) of about 3 and 5 mM were measured for hGXIIB and mGXIIB $sPLA_2$s (not shown). For comparison, a K_d value of 0.3 mM was found for hGXIIA. The very weak, but still significant binding of GXIIB $sPLA_2$s to the phospholipid vesicles, however, indicates that the proteins are most probably properly folded. Together, the low binding to phospholipids and the natural His-to-Leu

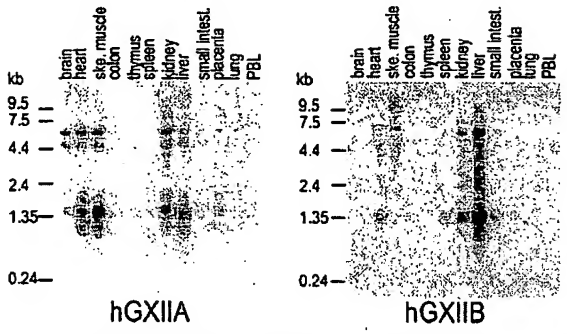

FIGURE 5: Tissue distribution of hGXII $sPLA_2$s. A human multiple tissue northern blot [2 μg of poly(A^+) mRNA per lane] was first hybridized at high stringency with a ^{32}P-labeled hGXIIB $sPLA_2$ riboprobe as described in Experimental Procedures. Abbreviations: ske. muscle, skeletal muscle; small intest., small intestine; PBL, peripheral blood leukocytes. The same blot was stripped and then hybridized at high stringency with the hGXIIA $sPLA_2$ riboprobe. Filters were exposed for 7 days at −70 °C.

active site mutation clearly explain why GXIIB $sPLA_2$s are not enzymatically active.

Binding Properties of Group XIIB $sPLA_2$s to the Recombinant Mouse M-Type Receptor Expressed in COS Cells. Previous studies have indicated that several mouse $sPLA_2$s are endogenous ligands of the mouse M-type receptor (*23*, *46*, *50*). We therefore addressed whether mGXIIB and hGXIIB could also act as ligands for this receptor. Competition binding assays with iodinated OS1, which is a specific ligand for the mouse M-type receptor (*51*), indicated that mGXIIB and hGXIIB do not bind to this receptor at concentrations as high as 100 nM (not shown). Because it was possible that GXIIB $sPLA_2$s bind to other domains within the large extracellular domain of the M-type receptor (*33*), while they do not compete for the OS1 binding site, we radiolabeled mGXIIB $sPLA_2$ and performed direct binding assays on COS cell membranes containing large amounts of the mouse M-type receptor. No specific binding was observed in these assays, suggesting that mGXIIB is not an endogenous ligand for the mouse M-type receptor.

Tissue Distribution of Group XIIB $sPLA_2$s. The tissue distribution of hGXIIB $sPLA_2$ was analyzed by hybridization at high stringency to a northern blot containing various human adult tissues (Figure 5). The same northern blot was then stripped and hybridized with hGXIIA to allow for a direct comparison of the tissue distribution. Transcripts for hGXIIB of about 1.4 and 6.5 kb are detected by decreasing abundance in liver, kidney, skeletal muscle, and heart. This tissue distribution appears to be different from that of hGXIIA, although some overlap could be observed. Indeed, the strongest level of expression for hGXIIA is found in skeletal muscle, followed by heart, kidney, and liver. A weak expression is also observed in brain and placenta, whereas no signal is observed for hGXIIB. Transcripts of similar sizes are observed for both $sPLA_2$s, which might be explained at least in part by the similar exon−intron structure of their respective genes. The northern blot tissue distribution of hGXIIB is in accordance with that estimated from the sources of the ESTs found in databases. Indeed, several ESTs were obtained from human liver and kidney.

The tissue distribution of mGXIIB $sPLA_2$ was analyzed by hybridization to a mouse northern and a RNA dot blot

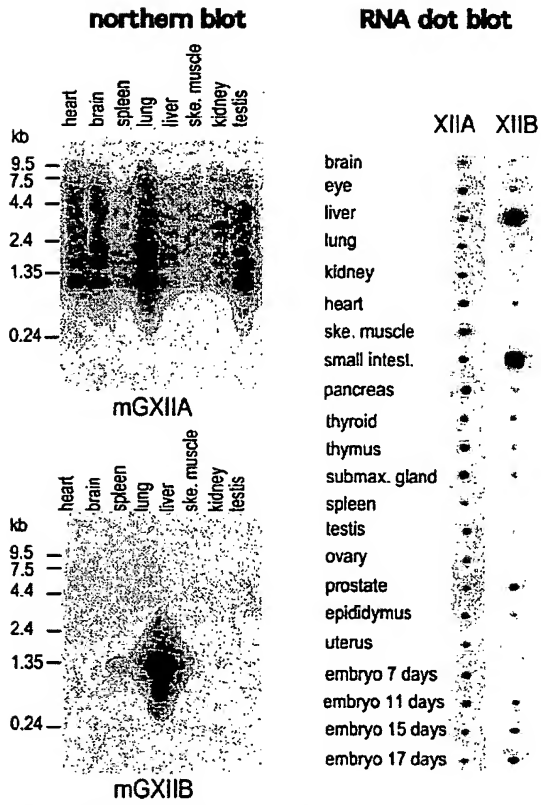

FIGURE 6: Tissue distribution of mGXII $sPLA_2$s. A mouse multiple tissue northern blot containing 2 μg of poly(A+) mRNA per lane and a master blot containing 100−500 ng of poly(A^+) RNA from mouse tissues were first hybridized at high stringency with a ^{32}P-labeled mGXIIB riboprobe as described in Experimental Procedures. Abbreviations: ske. muscle, skeletal muscle; small intest., small intestine; PBL, peripheral blood leukocytes; submax. gland, submaxillary gland. The same blots were stripped and then hybridized at high stringency with the mGXIIA $sPLA_2$ riboprobe. Filters were exposed for 7 days at −70 °C.

and then compared to that of mGXIIA hybridized to the same blots. A very strong expression of mGXIIB is observed in adult liver with a major transcript of about 1.3 kb and a minor one at 2.4 kb. Analysis of the RNA dot blot reveals that mGXIIB is also expressed at very high levels in small intestine and at lower levels in prostate, thyroid, and eye, among other tissues. A significant level of expression is also found during embryogenesis, and the signal is increasing upon development. This tissue distribution is in accordance with the identification of many ESTs from small intestine, liver, and embryos. On the other hand, mGXIIA is expressed from three transcripts, with two of them being similar in size with those of mGXIIB. The tissue distribution of mGXIIA is quite different, with a rather ubiquitous expression pattern in most, if not all, mouse tissues and highest expression levels in lung, heart, pancreas, and submaxillary glands. The wide expression pattern of mGXIIA is in accordance with that previously described by RT-PCR (*10*). Interestingly, the expression pattern of the two $sPLA_2$s during embryogenesis appears to be opposite (Figure 6).

All together, these results indicate that GXIIA and GXIIB $sPLA_2$s have different expression patterns in both mouse and human species, though some tissues express both $sPLA_2$s. Furthermore, as for other $sPLA_2$s (*29*−*31*, *48*), a distinct,

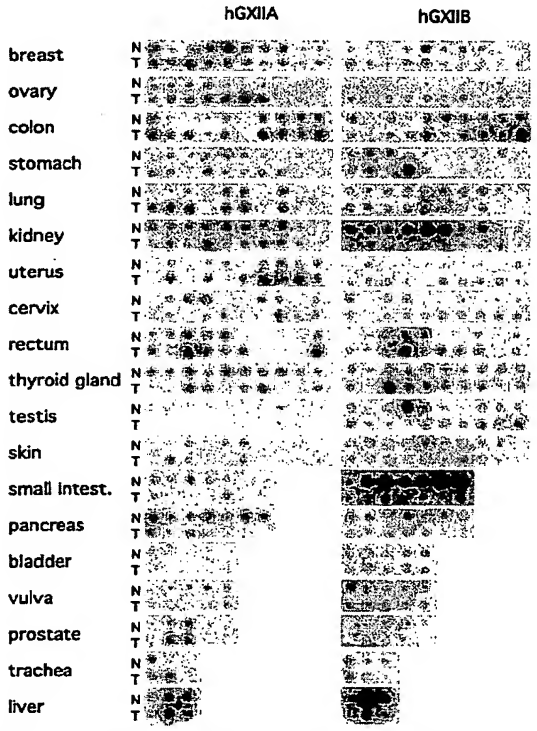

FIGURE 7: Cancer profiling array of hGXII $sPLA_2$s. A cancer profiling array II from Clontech containing 160 tumors (T) and their matched normal tissues (N) from individual patients was first hybridized with a ^{32}P-labeled hGXIIB riboprobe as described in Experimental Procedures. Abbreviation: small intest., small intestine. The same blot was stripped and then hybridized with the hGXIIA $sPLA_2$ riboprobe. Filters were exposed for 6 days at −70 °C.

yet overlapping tissue distribution is found for both $sPLA_2$s when mouse species is compared to human species. The mGXIIA $sPLA_2$ was previously found to be preferentially expressed in Th2 cells, suggesting that this $sPLA_2$ may be involved in the production of eicosanoids in these cells (*10*). It remains to be analyzed whether mGXIIB is also expressed in Th2 or Th1 cells.

hGXIIB $sPLA_2$ as a Cancer Marker. Several reports have shown that $sPLA_2$s including IIA, IID, and X are increased in human and mouse tumors (*28*, *52*−*57*, *58*, *59*), where they may play a role in providing arachidonic acid for subsequent prostaglandin E2 release. Alternatively, $sPLA_2$s such as IIA may exert protective effects against progression of colorectal and gastric cancers, as suggested for mouse group IIA (*26*, *27*) and very recently for human group IIA (*28*). Besides $sPLA_2$s, a decrease in the expression of the $cPLA_2$ group IV was also observed in both mouse and human tumors (*59*, *60*, *61*). It was therefore of interest to determine the expression status of hGXIIA and hGXIIB $sPLA_2$s in various human tumors and cancer cell lines. Figure 7 shows the expression patterns of the two group XII $sPLA_2$s in 160 tumors and their matched normal tissues from individual patients. A strong level of expression for hGXIIB is observed in the normal kidney, small intestine, and liver of several patients. However, the level of expression is dramatically decreased in nearly all of the corresponding tumors, indicating that hGXIIB is significantly downregulated during tumorigenesis in these tissues (Figure 7). In several cases, a

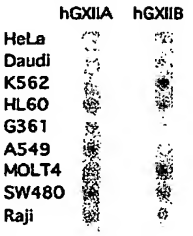

FIGURE 8: Expression of hGXII $sPLA_2$s in various human cancer cell lines. The cancer profiling array II from Clontech containing cDNA from the indicated cell lines was first hybridized with a ^{32}P-labeled hGXIIB riboprobe as described in Experimental Procedures. The same blot was stripped and then hybridized with the hGXIIA $sPLA_2$ riboprobe. Filters were exposed for 6 days at −70 °C.

decrease in hGXIIA and hGXIIB expression in testis and pancreas is also observed. On the other hand, a higher level of expression of hGXIIB is observed in some patients with tumors of the colon, stomach, rectum, and thyroid gland, although the significance of these data remains to be established, given the scatter in the expression data across the different patient samples. A detailed analysis of the hGXIIB promoter may reveal some clues to explain the tissue-specific expression patterns observed in these studies. Interestingly, hGXIIB expression was recently found to be significantly elevated in hepatitis C virus-associated hepatocellular carcinomas (*62*). Conversely, no dramatic upregulation or downregulation of the expression of hGXIIA could be observed in a whole set of tumor samples from the same tissue (Figure 7). However, as for hGXIIB, an increased or decreased expression was observed in the different tissues for some patients.

hGXIIB was also found to be expressed in a number of human cancer cell lines (Figure 8) including HL-60 (promyelocytic leukemia), Raji (B cell Burkitt's lymphoma), MOLT-4 (T cell lymphoblastic leukemia), A549 (lung carcinoma), and SW480 (colorectal adenocarcinoma). In contrast, we found either the absence of expression or only very low levels of hGXIIB in the following cancer cell lines: HeLa (adenocarcinoma), Daudi (B cell Burkitt's lymphoma), K-562 (myelogenous leukemia), and G-361 (skin melanoma). A dissimilar pattern of expression was found for hGXIIA, with the highest levels of expression observed in MOLT-4, SW480, HL-60, and K-562 cancer cell lines (Figure 8).

Concluding Remarks. This paper describes the molecular characterization of a novel human and mouse group XIIB $sPLA_2$ that belongs to the recently identified group XII collection (*9*, *10*). Interestingly, this $sPLA_2$ is present only in higher order organisms and is fairly highly conserved among different species, suggesting conserved and important functions. Most importantly, we demonstrated that this $sPLA_2$ is the first mammalian $sPLA_2$ of about 20 kDa that is completely devoid of enzymatic activity because of a mutation in the active site and very poor phospholipid-binding properties. In some regards, this protein can be considered as one mammalian "equivalent" of the various inactive snake venom $sPLA_2$-like proteins (*37*, *39*, *63*). Interestingly, the lipase family also comprises naturally occurring inactive forms (*64*−*66*), whose functions have not yet been determined. The tissue expression pattern of the GXIIB $sPLA_2$ is different from that of all other $sPLA_2$s including the homologous GXIIA $sPLA_2$, suggesting that this

sPLA$_2$-like protein has a function distinct from those of other sPLA$_2$ members. Its expression pattern is also different from that of otoconin-95, the atypical catalytically inactive sPLA$_2$-like protein that has been exclusively detected, to date, only in inner ears (*67*, *68*). Because the expression of hGXIIB sPLA$_2$ was found to be dramatically decreased in tumors of several tissues, it is tempting to speculate that the protein may act as a gene suppressor controlling tumor progression. Interestingly, the expression of the CC-10 protein (also called uteroglobin), which has been reported to act as an anti-inflammatory and antitumoral agent and possibly as a sPLA$_2$ inhibitor, is also known to be dramatically decreased in several tumors and cancer cell lines (*69*−*75*). The regulation of the expression of the GXIIB sPLA$_2$ during inflammatory conditions and in associated diseases will also be interesting to analyze in future studies. Finally, because of the absence of enzymatic activity, it is likely that the GXIIB sPLA$_2$ exerts its function(s) by acting as a ligand through binding to specific soluble or membrane proteins that remain to be identified (*1*, *33*). So far, we found that the GXIIB sPLA$_2$ does not bind to the well-known M-type receptor, but other sPLA$_2$ targets specific for this sPLA$_2$ remain to be discovered. The identification of such targets will certainly help to reveal the in vivo function of this particular sPLA$_2$.

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